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Use of Immunochemical Techniques for the Analysis of Pesticides*

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ABSTRACT

Immunochemical assays for small molecules such as pesticides are rapidly gaining acceptance among analytical chemists. These techniques are rapid, sensitive, cost effective and can easily cope with large sample loads. This review lists the advantages and disadvantages of the technique and describes the steps in assay development using examples from this laboratory, particularly the thiocarbamate herbicide molinate and the triazine herbicides. The focus is primarily on hapten synthesis strategies, assay format considerations, sample preparation and assay validation.

1 INTRODUCTION

There has recently been a heightened interest in developing immunoassays for pesticides and other environmental chemicals for residue analysis[1–7] even though the use of antibodies as analytical tools was first demonstrated many years ago.[8] The use of immunoassays for analysis of small molecules has been extensive in endocrinology, clinical chemistry and other fields. Application of immunoassay technology by environmental chemists is well behind these other fields, primarily because the early compounds of interest to these analytical chemists were more appropriately analyzed by gas-liquid chromatography.

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Although analysis by immunoassay is very similar to techniques commonly used in environmental chemistry, the disparate terminology is likely to make even an experienced analytical chemist hesitant to enter the field. A review by Van Emon *et al.*[9] describes the terminology used in pesticide immunoassay development, reviews the current literature in this field, and discusses the use of immunoassay in residue analysis.

The increasing efficacy of some new pesticides[10] has prompted regulatory agencies to seek lower detection limits (ng ml^{-1} and pg ml^{-1} levels). Growing concerns over possible adverse effects of long-term, low-level exposure to agricultural chemicals has led to more comprehensive crop and environmental monitoring programs. Coping with the large sample loads that can be generated from such regulatory programs is often difficult with current analytical techniques and analytical chemists are now exploring immunoassays as helpful analytical methods. There are many advantages to pesticide immunoassay such as specificity, sensitivity, precision, simplicity, cost effectiveness, speed and applicability. However, every technique has its disadvantages. The technology is new and reagents are not yet generally available. The method is subject to specific and non-specific interferences, and so, like GLC and HPLC, immunoassay requires confirmation by a more definitive method such as GC–MS. Finally it is not readily applicable to multi-analyte analysis. These factors are discussed in more detail by other reviews prepared in this laboratory.[4,11–13]

Compounds which have been analyzed by immunoassay include highly lipophilic organochlorine compounds such as aldrin, dieldrin,[14] endosulfan,[15] and dioxin;[16] organophosphorus compounds such as parathion[17] and paraoxon;[18] the highly insoluble *s*-triazine herbicides such as terbutryn[19] as well as the difficult-to-analyze paraquat;[20] sulfonylurea herbicides like chlorsulfuron,[21] volatile, unstable compounds like the thiocarbamate molinate,[22] and protein toxins such as the *Bacillus thuringiensis* toxins.[23,24] The biological activity of many pesticides may be confined to single geometric and chiral isomers, the different biological activities and metabolic or environmental fates of which make discrimination of their isomers imperative.[25–27] Immunoassay technology can be applied to most compounds including many which are difficult to determine by classical analysis. Only very small molecules or very hydrolytically-unstable molecules are inappropriate targets. Difficulty of synthesis of the appropriate hapten may also be a limiting factor as discussed below.

Immunoassays can be rapid and highly cost effective. Assays have been developed which minimize sample cleanup[22,28,29] and much of the assay can be automated easily with initial instrumentation costs being very low.[30] Among currently available analytical technologies, immunoassay is the most easily adaptable to the analysis of large sample loads. This may be very important in the environmental area because of the need to analyze large numbers of samples to obtain high confidence estimates of low contamination rates.[13]

There are a number of steps to the development and implementation of an immunoassay. This paper will deal with the development of an assay, primarily focussing on the steps of hapten synthesis, assay format and validation, as well as some of the pitfalls and problems still to be faced.

2 DEVELOPMENT OF AN IMMUNOASSAY

The steps in immunoassay development include selection of hapten, synthesis and characterization of hapten, covalent binding to carrier molecules such as proteins, immunization, purification and characterization of antibody, development and optimization of antibody, development and optimization of format, application to field samples and validation. For the chemist, working with proteins may appear as a major stumbling block. For the biologist the preparation of the hapten provides a similar challenge, although easily-prepared haptens can be designed for many molecules. For many other steps, routine techniques work as well as highly innovative chemistry or biochemistry.

2.1 Criteria for hapten selection

Pesticides of small size (< 1000 daltons) must first be covalently linked to a larger molecule or carrier, usually a protein, to produce an immune reaction.[31] The 'perfect' hapten contains as much of the structure of the target compound as possible plus a handle to facilitate recognition of the target structure by antibodies. This is usually 3 to 6 atoms long and contains a functional group ($-NH_2$, $-COOH$, $-OH$, $-SH$) which can be covalently linked to a protein. If the target molecule has no reactive group at all, derivatization procedures are required to yield an appropriate spacer arm as well as a reactive functional group for conjugation to the protein.

One lacks the ability to predict exactly what an antibody population will recognize, but specificity generally depends upon steric properties plus hydrogen bonding and dipole-dipole interactions.[32] There are numerous possible ways to attach a single handle of a hapten to a carrier protein. Solubility is an important criterion in the selection of this handle, which, in most cases, should be as non-polar as possible, containing no functional groups other than that necessary for coupling. This minimizes handle recognition in antibody formation. In some cases a polar handle may help in hapten presentation. The synthesis of the hapten can be performed in whatever solvent is most appropriate. However, the coupling of the hapten to the carrier protein is generally performed in aqueous solution and antibody production takes place in an aqueous environment in the animal. Thus, the chemistry which links the hapten to the carrier molecule must be hydrolytically (biologically and chemically) stable for a moderate period of time.

Probably the first practical criterion in hapten selection is the ease of synthesis. During the development of a new pesticide, hundreds of related structures are commonly synthesized in order to arrive at the optimum structure and to gain patent protection. It would be beneficial at this point to synthesize several additional analogs closely related to the prime candidate for commercial development as potential haptens. This could eliminate a major part of the lead time required for assay development, with a resultant reduction in analytical costs and might therefore be a valuable corporate policy.

Potentially useful haptens are also obtained during studies of the metabolism of the target compound. For example, fenpropimorph and diclofop-methyl metabolites

possess a carboxylic acid group and could be coupled readily to a carrier protein, while still retaining enough of the parent structure for antibody recognition.[33,34]

The current program in this laboratory emphasizes four major factors in hapten synthesis: (1) position of spacer arm to maximize exposure of unique portions of the molecule which should control recognition; (2) length of the spacer arm; (3) polarity (or lack thereof) of the spacer arm and (4) functional group variations for ease of attachment to protein carriers and enhanced hapten density.

With the herbicide molinate, only complicated synthetic routes could be envisaged for attachment of the spacer arm to the hexahydroazepine ring of the molecule (**I**; Fig. 1), and so attention was concentrated on the sulfur end of the thiocarbamate. Haptens with spacer arms of various lengths using methylene groups (**IA,B**) and *p*-aminophenyl groups (**IC,D**) were prepared.[22]

Thiobencarb and EPTC (Fig. 1; **II** and **III**) are two other thiocarbamates with which the strategy of sulfur-substitution spacer arms bearing functional groups has been used, as with the molinate example. However, since there are many thiocarbamates that are very closely related structurally at the nitrogen end of the molecule, a more extensive and complex route was pursued to obtain a hapten with a spacer arm on the nitrogen end of the thiocarbamate thiobencarb (Fig. 1; **IIA**). This hapten is expected to produce exceptionally specific thiobencarb antibodies as it leaves exposed the most specific portion of the thiobencarb molecule, the *p*-chlorobenzylthio moiety.

Current studies with triazines have also incorporated variations adapted from the thiocarbamate hapten synthesis. Carboxylic acid derivative haptens of atrazine and simazine at two positions have been investigated (Fig. 2). Replacement of the chlorines of atrazine or simazine with mercaptopropanoic acid have provided haptens which produced class-specific antibodies. Haptens with hexanoic spacers at

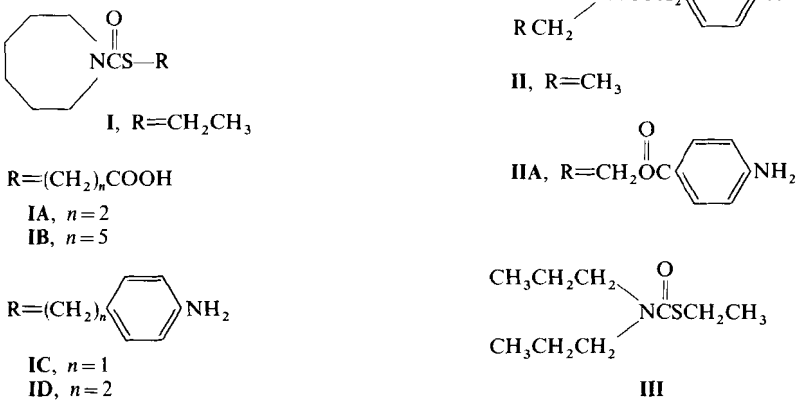

Fig. 1. Structures of some thiocarbamates. **I**, molinate; **IA**, *S*-2-carboxyethyl azepane-1-carbothioate; **IB**, *S*-5-carboxypentyl azepane-1-carbothioate; **IC**, *S*-(4-aminobenzyl) azepane-1-carbothioate; **ID**, *S*-2-(4-aminophenyl)ethyl azepane-1-carbothioate; **II**, thiobencarb; **IIA**, 4-aminobenzoate ester of *S*-(4-chlorobenzyl)ethyl (2-hydroxyethyl)thiocarbamate; **III**, EPTC (*S*-ethyl dipropylthiocarbamate).

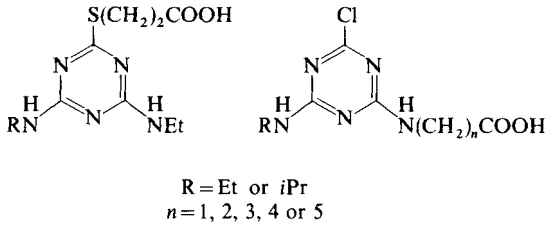

Fig. 2. Structures of some triazine haptens synthesized in this laboratory.

the 4- or 6-(alklamino) positions have produced more compound-specific antibodies. The effect of varying chain length at the 4-position is also under investigation.

2.2 Covalent binding to carrier molecules

A very pragmatic and historically successful approach used in this laboratory is to prepare a chemically-activated hapten in rather large amounts in a dry, water-miscible organic solvent. Aliquots of this active hapten are added to three or four different proteins in at least two hapten protein ratios. After dialysis, these solutions can be divided up for long-term storage, giving the analyst a repertoire of proteins with different hapten densities to use for immunizing and coating antigens.

It is often not appreciated by biochemists that many water soluble proteins can tolerate relatively large amounts of organic co-solvents during this coupling procedure without denaturation or that denaturation of a carrier protein, with no function other than providing antigenicity, may even be beneficial, as long as solubility is retained. Especially for lipophilic haptens, very high concentrations of co-solvent may dramatically improve coupling. Just as the pH used for coupling is a compromise among the optima for reaction rate, hapten stability and protein stability, the temperature used can be a compromise between the high temperature that accelerates coupling and the low temperature which retards protein denaturation (melting) in organic co-solvents.

The functional group of the hapten governs the selection of the method to be used to conjugate the hapten to the carrier. Two procedures routinely used for conjugation of carboxyl-containing haptens to proteins are the mixed anhydride procedure, originally developed for peptide preparation,[35] and methods utilizing carbodiimides.[36,37] The mixed anhydride method has been used for benzoylphenylureas,[28] thiocarbamates[22] and chlorinated biphenyls.[38] As examples of the water-soluble carbodiimide method, Newsome and Shields[39] coupled 2-succinamidobenzimidazole to human albumin at pH 7 and Wie *et al.*[28] reported the coupling of five benzoylphenylureas to several carrier proteins at pH 6·5. A somewhat more elegant procedure involves the use of a carbodiimide to synthesize an active ester which is then added to the protein with or without prior isolation.[40] Wing *et al.*[41] prepared the active ester of *S*-bioallethrin hemisuccinate, which was coupled to several proteins and also to tyramine for radiosynthesis and structural proof of conjugation. This technique has also been used for current work with

triazines in this laboratory, for dieldrin,[42] fenpropimorphic acid,[33] maleic hydrazide[43] and endosulfan.[15]

Haptens containing amine groups can be conjugated by a simple diazotization as was done for molinate.[22] Hydroxyl-containing haptens can be conjugated to proteins directly after derivatization of the protein with succinic anhydride.[44] Sulfhydryl containing haptens may be conjugated through homo- or heterobifunctional reagents.[45]

Some immunoassay formats require enzyme-labeled haptens or enzyme-labeled antibodies. The procedures mentioned above can be also used for these conjugations.[19,33,34] These and other coupling procedures have been extensively reviewed.[45-48]

2.3 Antibody production

This step in assay development includes immunization, purification and development and characterization of antibodies and complete discussion is outside the scope of this paper. The following however, are some useful generalizations.

Essentially any vertebrate can be used as a source of antibodies. The rabbit offers the advantages of being easy to care for, and it produces a moderate amount of serum, often with high titer and is thus widely used. Monoclonal antibody technology, originated by Köhler & Milstein in 1975,[49] makes it possible to establish cell lines that produce a single desired antibody indefinitely *in vitro*. Hybridomas require much more time, labour, and expense to prepare than antisera, but each monoclonal antibody is a reagent with a single defined affinity and specificity, and it can be made in unlimited quantities as long as the hybridoma line is maintained in culture or in storage.

Regardless of whether polyclonal sera or monoclonal antibodies are sought, the antibody response to a given antigen depends on the characteristics of the conjugate, the animal's immune system, and the immunization schedule and methods. Williams & Chase[50] and Vaitukaitis[51] describe a number of immunization procedures and schedules, many variations of which are widely used. In this laboratory, for the production of polyclonal antibodies in rabbits, multiple intradermal injections are made along the back of the animal. An initial series of injections is followed by booster injections some weeks later. The animal is bled after each boost and the characteristics of the serum determined. One can either continue to collect, and possibly pool, sera following numerous booster injections or bleed the animal out. However there is no standard protocol for immunization and most approaches are largely empirical.

In general, monoclonal antibody production can be split into 4 major tasks: (a) immunization, (b) cell fusion and primary selection, (c) postfusion cell management and secondary selection and (d) expansion and scaled-up antibody production. Numerous descriptions of the general procedures for generating monoclonal antibodies have been published.[52-54] Optimal conditions, which can aid in the prediction of the success of production of the desired monoclonal antibody, are generally determined empirically.

2.4 Immunoassay format

Once an antibody to a pesticide is obtained, it can be used in a variety of formats which can result in rapid, qualitative field procedures as well as highly-quantitative laboratory procedures. Each of these formats has unique advantages in terms of speed, cost, sensitivity and other factors. All the formats used for pesticide analysis share three components: specific antibody, conjugated hapten, and target analyte. The basis for measurement of the analyte is the competition of the analyte with the binding of specific antibody to conjugated hapten, based on the structural similarity between the analyte and the conjugated hapten. These interactions are governed by the Law of Mass Action. The differences among immunoassay formats lie in whether separation of bound and free antibody is required, and in the nature of the detection system. A number of analytical techniques can be used in the assay detection system, including radioactivity, turbidity, polarization of light, visible or ultraviolet absorbance, fluorescence, phosphorescence, chemiluminescence, bioluminescence or electron spin resonance.

Table 1 lists the currently reported immunoassays for pesticides, some of the first of which utilized radio-immunoassay techniques.[17,42] The introduction of enzyme immunoassay[55,56] has led to the gradual replacement of isotope labels by enzyme labels. The format presently favored for pesticide immunoassay is the competitive ELISA or enzyme linked immunosorbent assay. One form of competitive ELISA is the immobilized antigen assay,[22,29] which is based upon competition between the immobilized antigen and an unknown and variable amount of soluble analyte (sample) for a small fixed amount of soluble antibody. The concentration of the analyte in the sample is measured indirectly by the quantitation of bound antibody after it is separated from the free antibody (Fig. 3).[57] After optimization of this assay format for molinate,[22] extensive characterization of the assay by Harrison *et al.*[58] gave a limit of reliable measurement of 21 ng ml^{-1} and 50% inhibition of the assay at 106 ng ml^{-1} molinate.

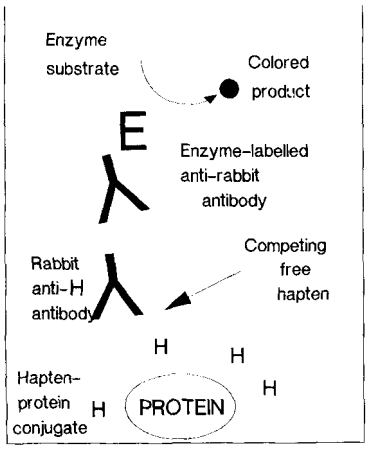

Fig. 3. A standard competitive ELISA format. A coating antigen is bound to the solid phase. Hapten, in sample or standard, competes for a fixed amount of antibody. Antibody not bound by hapten binds to the antigen on the solid phase. This bound antibody is quantitated by binding an enzyme-conjugated second antibody against the first antibody IgG. Substrate is added and color formation monitored. The more hapten in the sample, the less antibody is available to bind to the plate and thus less second antibody binds and less enzyme is present for color development.

TABLE 1
Pesticides for which Immunoassays have been Developed

Pesticide	*Reported detection limit*	*Method*	*Reference*
Fungicides			
Benomyl and metabolites	0·1 ng	FIA	72
	1·25 ng	RIA	39
	350 ng ml^{-1}	ELISA	73
Metalaxyl	63 pg ml^{-1}	ELISA	74
Triadimefon	1·0 ng ml^{-1}	ELISA	75
Fenpropimorph	13 pg ml^{-1}	ELISA	33
Herbicides			
Paraquat	10 ng, 0·1 ng	RIA	76
	0·1 ng	ELISA	29
2,4-D, 2,4,5-T	13 ng	RIA	77
	100 pg	RIA	78
Diclofop-methyl	23 ng ml^{-1}	ELISA	34
Terbutryn	4·8 ng	ELISA	19
Atrazine	0·1 pg ml^{-1}	ELISA	79,80
	1 ng ml^{-1}	ELISA	81
Chlorsulfuron	0·1 ng ml^{-1}	ELISA	21
Molinate	3·0 ng ml^{-1}	ELISA	22
Insecticides			
Diflubenzuron	3·9 ng	ELISA	28
Parathion	4·0 ng	RIA	17
Paraoxon	25 ng	ELISA	18
Bioallethrin ((*S*)-cyclopentenyl isomer)	0·5 ng	ELISA	41
Dieldrin	150 pg	RIA	14
Endosulfan	3·0 ng ml^{-1}	EIA	15
Chlordane	5·0 ng ml^{-1}	EIA	82
Aldrin	700 pg	RIA	14
Aldicarb	300 ng ml^{-1}	EIA	83

Another ELISA format was used for the fungicide fenpropimorph,[33] utilizing an immobilized antibody to capture the analyte-specific rabbit antibody. It is based on an equilibrium reaction between antibody, hapten and hapten-enzyme labeled conjugate (Fig. 4). This technique was compared with the immobilized antigen ELISA for molinate. Molinate hapten (Fig. 1; **IB**) was conjugated to an enzyme label and the same antibody as that used in the immobilized antigen assay was employed. This assay configuration offered several significant advantages over the immobilized antigen competitive ELISA (Fig. 3). An antimolinate antibody dilution of only $1:10^5$ was needed with this technique in contrast to a $1:4 \times 10^3$ dilution needed for the immobilized antigen technique. At the same time the sensitivity improved by a factor of 5 from 106 ng ml^{-1} to 20 ng ml^{-1} for the concentration of molinate needed to inhibit the reaction by 50%. In addition, the

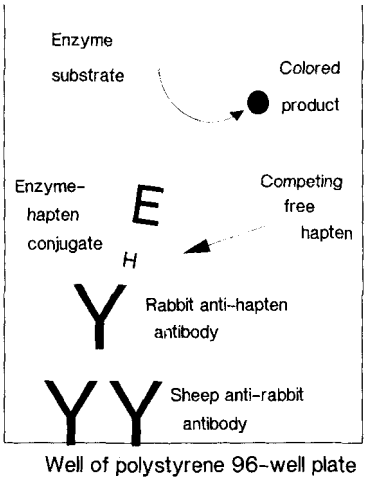

Fig. 4. A double antibody ELISA technique. Anti-*rabbit sheep IgG is bound to the solid phase. The* binding of the specific antibody to the sheep IgG and the competitive immunoreaction between hapten and hapten-conjugated enzyme for the specific antibody occur simultaneously. The color conversion is indicative of the amount of enzyme-labeled hapten that is bound to specific antibody captured by the solid phase-bound antibody.

assay was simpler and less time-consuming because the number of steps was reduced.

These results were in agreement with similar work by Meyer[59] and clearly point out that it is primarily the antibody that determines the assay sensitivity, although prudent selection of assay format may improve sensitivity. This is true regardless of whether the label is a free radical, enzyme, coenzyme, enzyme inhibitor, virus or a fluorescent, phosphorescent, chemiluminescent or bioluminescent molecule. However, most immunoassays fail to exploit the level of sensitivity possible with the antibody used. In such situations, improved sensitivity can be obtained in many ways, but most obviously by selection of the label. For example, some of the above mentioned labels can be detected at concentrations as low as 10^{-16} M. Label detection limits as low as 10^{-20} M can be obtained through fluorophor or luminophor techniques.[60] Useful comprehensive reviews for the various assay formats have been published.[45,61-63] Some of these formats are available commercially as kits for a few pesticides and environmental chemicals.[3] Once the optimum sensitivity dictated by the average K_d of the antibody is obtained, one can only improve sensitivity further by improving the reliability of measurements (probably by making numerous rapid measurements which will be averaged electronically), and by reducing assay volume. These two approaches will be exploited in the coming decade as biosensors allow repeated assays on very small volumes.

2.5 Sample preparation

Sample preparation methods can be as simple as taking an aliquot from a sample collected for other methods. However, stabilizers and salts sometimes added for classical analysis can denature or interfere with the antibody. More typically, sample preparation methods will need to be developed specifically for use with immunoassay. Extraction methods that can handle larger numbers of samples of much smaller size are needed. Highly-volatile solvents or water-miscible solvents

are the most desirable, since immunoassays ultimately are run predominantly in aqueous solution.

An example of the evolution of sample preparation methods can be illustrated with work on molinate in this laboratory. For GLC analysis, molinate is usually extracted from water with toluene but large concentrations of toluene cannot be added directly to an immunoassay. First efforts involved simply evaporating the toluene under a nitrogen blanket or reducing the volume in a centrifugal vacuum evaporator so that the antibody could be added directly to the residue. Due to the volatility of molinate, both of the above procedures resulted in high and variable sample loss. The solution to these difficulties was to use a small amount of non-volatile propylene glycol to trap the molinate during evaporation of the toluene, resulting in minimal sample loss. Antibody could then be added directly to the propylene glycol although, in practice, a small amount of acetonitrile was added to the propylene glycol to decrease viscosity and facilitate measurement of small aliquots for addition to antibody.

Other more volatile solvents and solvent combinations were also investigated as alternatives to toluene extraction. The highly-volatile pentane was useful, but was so non-polar that it penetrated poorly into sample matrices. A second, more polar solvent, dichloromethane, was added to the pentane and both solvents evaporated onto a bed of propylene glycol following extraction. Recoveries of [^{14}C]molinate by this method improved from 66·9(±3·2%) to 94·7(±0·2)% as the pentane: dichloromethane ratio was altered from 8:2 to 0·5:9·5.[22] The small amount of co-solvent remaining in the propylene glycol following volume reduction in the centrifugal vacuum evaporator did not disrupt the immunoassay. Pentane, however, proved to be too volatile, as the volume of samples extracted for ELISA was only 1 ml. Instead, a mixture of ethyl acetate and hexane was used as a compromise between the desired polarity, volatility and penetrating ability. Again, propylene glycol was added as a trapping solvent. Since antibodies are sensitive to ethyl acetate, a very small portion of ethyl acetate + hexane (1 + 9 by volume) was used and 100 g litre^{-1} aqueous salt solution added to improve the recoveries. Such simple partitions can yield a major purification and can be performed on very small scale for immunoassay analysis.

The partitions described above were simple and inexpensive, but it seems likely that solid phase extraction systems will dominate sample preparation for immunoassay. Solvents less harmful to antibodies, such as methanol or acetonitrile, can be used as eluants, or the solvent used can be removed by evaporation. For example, comparable recoveries were achieved with ethyl acetate or methanol when eluting molinate from C_8 cartridges.[64] Comparisons were also made between liquid-liquid extraction and solid phase extraction for 'spiked' and field samples of air, water, and soil. Solid phase recoveries were similar to those from liquid-liquid extraction as measured by both GLC and ELISA. In addition, there was excellent agreement between results for air or water samples analyzed by GLC or ELISA, but soil samples in the ELISA exhibited some matrix effects.[64]

Antibodies can often tolerate high concentrations of organic co-solvents such as ethanol, dimethylsulfoxide, acetonitrile, tetrahydrofuran, dioxane, methanol and propylene glycol. These solvents can enhance the solubility of the analyte, remove

the analyte from surfaces, disrupt lipid micelles in the sample matrix, and increase ease of sample handling. The properties of antibodies vary widely and a concentration of organic solvent that has no effect on one immunoassay can dramatically decrease or even increase the sensitivity of other assays.[22] In general, immunoassays of lipophilic compounds which contain at least a few percent of organic co-solvent appear to be more resistant to matrix effects.

2.6 Assay validation

A general approach to the problem of analytical method validation has been summarized by Horwitz[65] and is applicable to such immunoassay methods as the determination of molinate in surface water. A crucial, but often neglected, aspect of immunoassay validation is intralaboratory optimization, which should always include careful evaluation of the quality of individual components of the assay system, an aspect not stressed by Horwitz. Our experience has been that two of the most critical of these immunoassay components, which are often ignored, are the microplates (or other solid phase) and the automatic 96-well microplate readers used for many ELISA methods. The quality of commercial plastic products varies greatly and 96-well microplates are no exception.[66] Even using one of the more expensive, consistent and better quality microplates, the molinate study showed that inter-well variability within plates was the largest single contributor to total assay variability (Harrison *et al.*[58]). Also, because the nature of the solid phase adsorbent (i.e. antibody or synthetic coating antigen) may vary widely, it is essential to evaluate the solid phase quality independently for each assay. It is also prudent to monitor product lots for lot-wise variability. Quality control for microplate readers is somewhat simpler and one aspect of it has been scrutinized in this laboratory.[67] The price of neglect of these areas can be extremely high and these factors need careful consideration in the optimization process.

Assay validation also involves comparison of the immunoassay with an established method, and when making these comparisons, several items must be considered. Immunoassays often require less sample preparation time than conventional methods. Thus analysts must make an effort to analyze samples by both methods in a timely fashion so as to avoid complicating factors such as biotransformation, volatilization, chemical degradation, or other time-dependent changes to the matrix. Differences in degree and structure of replication, intra- and inter-assay variation, differences in detection limit and calibration curves used for data calculation need to be taken into account when comparing the results of the two methods. It is also important to note that the low cost and ease of processing larger numbers of samples and replicates may lead to superior reliability of immunoassay results. For example, in the molinate field study comparing ELISA and GLC, the ELISA results consisted of quadruplicate determinations on each of three dilutions per sample, while the GLC analysis was not replicated at any level (Harrison *et al.*[58]). When comparing the two methods, appropriate statistical tests must be applied.[68]

This laboratory has been working with groups such as the Association of Official Analytical Chemists (AOAC) and the US Environmental Protection Agency (EPA) in the official validation process to develop well-defined validation protocols

suitable for general immunoassay use. These protocols must deal realistically with matrix effects. In the absence of interfering substances, the standard curve in buffer is parallel to the curve obtained by diluting the sample.[69] Deviations from parallelism with the standard curve may result from non-specific interferences. Slope comparisons using linear regression of logit-log transformed data provide a simple test for matrix effects. Alternatively, a sample may be split and one part spiked with a known amount of analyte. Failure to show additivity, in this case, is also an indication of an inhibitory effect, probably due to the matrix. Thus, demonstration of standard curves in the matrix of interest is important, but is not sufficient to predict success of the method, especially for environmental samples, such as soil, that may vary widely. A validation study consisting of only a few samples may fail to uncover errors resulting from interference with the assay. Biotransformation of the target and variability of matrix effects among samples must be evaluated in the analysis of field treated samples.

The linear standard curves familiar to gas chromatographers are easily generated by linear regression and determination of sample concentration is therefore quite simple. Most immunoassay standard curves are inherently sigmoidal and this may be perceived as a problem by some analytical chemists. Microplate readers can be effectively interfaced with computers to collect and analyze such data. Most commonly, plate readers come equipped with software based on curvilinear fitting such as logit and least squares which are used to linearize and analyze EIA data.[70]

A thorough approach to validation was demonstrated in a recent study using an ELISA for molinate for the analysis of water samples from a treated rice field (Harrison *et al.*[58]). Samples were analyzed by GLC and ELISA and comparisons were made of relative sensitivity and precision of the two methods, as well as inter- and intra-assay variability. The limit of detection with GLC was 1 ng ml^{-1}, while the ELISA had a limit of reliable measurement[71] of 21 ng ml^{-1}. The ELISA and GLC methods were equally precise although the ELISA method had a slight high bias and GLC a slight low bias. Although these comparisons are necessary, it is very important to realize that GLC data was obtained on toluene extracts of the water samples, while in the ELISA, water samples were buffered and analyzed without further workup.

3 CONCLUSIONS

Many aspects of immunoassay development and validation have been introduced here. Many of these have been examined in detail by other reviewers. The field of immunoassay is constantly changing as scientists from the areas of analytical chemistry, synthetic chemistry, clinical chemistry, and immunology combine their expertise for the application of immunoassay to pesticides and environmental chemistry, synthetic chemistry, clinical chemistry and immunology combine their validation toward routine use for pesticide and environmental analysis. Once the practical steps required for complete method validation are established, several new questions will arise. Will monoclonal antibodies replace polyclonal antibodies? How will antibodies be made available for general use (commercially or through antibody banks)? Should these assays be delivered in standardized 'kits'? Should

the official validation process consider kits or components? How can this technology be transferred successfully to analytical laboratories? These questions will be answered during the on-going process of establishing immunoassays as analytical methods. As described above, immunoassays are generally applicable, flexible, cost effective, and simple to use; there is no doubt that they will play an important role in the analytical chemistry laboratory of the future.

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